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Open and closed domains in the mouse genome are configured as 10 nm chromatin fibres

Eden Fussner, Mike Strauss, Ugljesa Djuric, Ren Li, Kashif Ahmed, Michael Hart, James Ellis and David Bazett-Jones

Corresponding author: David Bazett-Jones, Hospital for Sick Children

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 April 2012

Thank you for the submission of your research manuscript to EMBO reports. I have now had the opportunity to read and to discuss it with my colleagues here. I regret to say that we all agree that the manuscript would not be suitable for EMBO reports.

We note that your study reports that 30 nm chromatin fibers cannot be observed in vivo. By combining electron spectroscopic imaging (ESI) and EM-tomography, you visualize chromatin of cultured mouse fibroblasts and mouse lymphocytes and liver cells in 3D. This shows that only 10 nm chromatin fibers are present in both open and compact chromatin structures, while 30 nm fibers can be observed in starfish sperm nuclei.

We appreciate that your findings support the view that 30nm chromatin fibers do not exist in vivo, and we think this will be of interest to researchers in the field. However, we receive many more manuscripts than we can publish, and thus have the difficult task of selecting those we think most likely to compete successfully with other submissions during the in-depth review process. In this case, we note that it has been reported before that a 30 nm chromatin fiber cannot be observed in human mitotic cells. While we recognize the application of ESI together with EM-tomography, and that you extend the previous findings to both open and compact chromatin structures, we still think that the earlier publications impact on the novelty of your findings. We have therefore decided not to

proceed with in-depth review.

In the interest of your manuscript and your time, I am providing you with an editorial decision on your manuscript that will allow you to submit it elsewhere without further delay. I am sorry to disappoint you on this occasion, and I thank you once more for your interest in our journal.

Yours sincerely,

Editor
EMBO Reports

Correspondence - Appeal 30 April 2012

Thank you for your prompt reply. I wish to re-state why I think this paper deserves a critical review. First, the inability to detect 30 nm fibres in mitotic chromosomes could be due to the fact that the extremely close spacing of nucleosomes in metaphase chromosomes prevents the detection of a larger spacin, or the 30 nm fibres simply unfold due to the close packing of the nucleosome subunits. This reality means that "believers" in the 30 nm fibre will not be convinced that the Maeshima result has disproved a 30 nm fibre in any meaningful way. Second, the transition between 10 and 30 nm fibres as a regulatory mechanism has been a cornerstone of molecular biology for decades. Showing that there are no 30 nm fibres in a variety of chromatin densities in interphase severely damages this old model. The Maeshima paper, a mitotic study, cannot address this issue since the same regulatory mechanisms do not operate in mitosis.

I would be happy if you would reconsider your decision.

Yours sincerely

2nd Editorial Decision 22 May 2012

Thank you for the submission of your manuscript to EMBO reports. We have now received three referee reports on your study that are copied below.

As you will see, while the referees agree that the findings are potentially interesting, they also all point out that the size of the chromatin fibers/nucleosomes is not clear, and that quantitative data/images need to be provided. Scale bars certainly need to be included in all images. All three referees are also concerned about the possibility that the mouse chromatin structure was disrupted during sample preparation, which must be addressed, or better, excluded.

Given these evaluations, we would like to invite you to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #2:

In the manuscript entitled "Open and closed domains in the mouse genome are configured as 10 nm chromatin fibres", the authors the ongoing controversy concerning the existence of the 30 nm chromatin fiber in mouse cells. Upon careful examination of both cultured mouse embryonic fibroblasts and cells extracted from murine tissues using ESI-EM pioneered by the senior author, the authors report that they exclusively observe 10 nm fibers, even heterochromatic domains such as chromocenters. This holds true whether they examined chromatin within murine cells in culture, or within issues extracted from various organs in the mouse. Furthermore, the authors show that they can observe 30 nm fibers in starfish sperm nuclei, providing evidence that their method analysis does not disrupt putative higher order chromatin compaction. This concise report offers a valuable insight into the chromatin conformation inside living tissues, and provides further argument to the ongoing debate of whether the 30 nm higher order chromatin fiber actually exists in mammalian cells.
However, there are 3 major concerns that should be addressed to bolster the case presented by the authors:
1) The main focus of the article is the presence or absence of a chromatin fiber of certain dimensions, but no quantitative data is provided in the manuscript. Given the excellent quality of ESI-electron tomography figures presented by the authors, and the availability of 30 nm fiber control (starfish sperm), a sample image with actual measurements should be included, alongside graphs or tables outlining the actual numbers and statistical analysis of a significant number of measurements. This would allow readers to gain a fuller appreciation of the exclusive presence of 10 nm fibers in analyzed samples.
2) Similarly, due to the quantitative nature of the claim, the readers must be allowed to critically evaluate each image presented in the manuscript in context of the size of fibers. This can be achieved by consistent use of clearly labeled scale bars. However, figures 1 and 4 only provide scale bar in single panels, while figures 2 and 3 omit the scale bar entirely. The authors should include clearly labeled scale bars of relevant sizes (ex. 10nm or 30nm) in each panel to let the readers easily identify the dimensions of structures presented in the figures for themselves.
3) A significant technical concern is that although the starfish retains its 30nm chromatin fiber under these sample preparation conditions: after all, this organism lives in harsher oceanic environmental conditions than the mouse. Mammalian nuclear membranes might be more sensitive to manipulation such as freeze/drying, so that the 30nm chromatin fiber collapses globally in mouse nuclei. The authors should provide a strong clear argument for why they don't think this is an explanation for their results. Alternatively, they should present additional data from other species (say plants, or yeast or worm etc), which also don't (or do) demonstrate 30nm fibers. This will robustly and unequivocally make the point that 30nm fibers are indeed not a dominant organizing principle in the mouse nucleus.

Minor concerns:
- It should be clarified in the abstract that the findings of the paper show exclusive presence of 10nm fibers "in mouse", as that was the subject and title of the study.
- In the methods section the authors describe immunofluorescent staining using H3K9me3-specific primary antibody and Cy3-labeled secondary antibody to identify heterochromatin rich regions. The reviewer would like to see an image of such staining correlated with ESI image, showing that the region analyzed is indeed enriched in this mark, as claimed in the manuscript (Paragraph 2 of Results and Discussion).
- Also in paragraph 2, the authors mention the exclusive presence of 10 nm fibers in open and closed domains, and reference Figure 1. To that end, a magnification of the open chromatin domain, similar to the one provided for closed domain, would be helpful.
- For clarity, supplemental figures should be referenced in the manuscript in order, i.e. Supplemental Figure 1 before Supplemental Figure 2.
- On page 4, the authors mention 30 nm chromatin fiber in starfish sperm nuclei, and reference Supplemental Figure 2A-C. However, these figures are of mouse cells, namely mouse embryonic fibroblasts, lymphocytes, and liver cells, according to the figure legend. Did the authors mean Figure 1C of the manuscript instead?

- Figure 3 legend is misleading. Digitally zoomed images are on the right (not left), and side views are on the left (not right).
- On page 5 the authors mention that lymphocytes have high representation of compact chromatin domains throughout the nucleus. This should be supported with an appropriate reference. Likewise, the mention of equal representation of dispersed and compact chromatin domains in liver cells should also be accompanied by a reference.
- On page 5 the authors discuss open and compact chromatin domains in MEF cells, but reference Figure 1C, which shows starfish sperm nucleus.
- The claim in the last paragraph that "interconversion of 10 nm to 30 nm chromatin fibres does not represent a major gene regulatory mechanism, and is not required for regulation" should be supported by an appropriate reference.
- (Optional) The authors mention they observed 10nm fibers in the somatic cell nuclei of starfish testis. Can the authors show an image of that?

Referee #3:

Chromatin structure is important to many biological problems and is of interest to a broad audience. The 30-nm fiber models of chromatin folding have been accepted by most cell biologists. In the past few years, however, the generality of the 30-nm-fiber has been challenged by studies of chromatin structure in situ. In this paper, the authors used electron spectroscopic imaging in combination with tomography to study the 3-D organization of chromatin in mammalian cells. ESI tomography is a novel and powerful technique because it provides 3-D chemical information, namely the distribution of the cell's phosphorus-rich components. The authors conclude from their image analysis that the organization of both "open" and "closed" chromatin is the "beads-on-a-string" 10-nm fiber - not the 30-nm fiber. From an electron microscopy point of view, the most significant claim is that the path of linker DNA could be determined. Therefore, the authors' present work could be a rich platform for future studies on chromatin folding and transcriptional regulation in situ.
My initial enthusiasm was curbed, however, by two major concerns. First, how was the chromatin width measured? The nucleosome is ~10 nm in diameter, so I would expect the ESI densities to be 8 - 12 nm wide at maximum, taking into account inaccuracies in magnification calibration. To my surprise, I consistently measured the chromatin dimensions to be 15 - 25nm using the authors' scale bars in figures 1 and 4. The scale bar in figure 1 appears to be correctly labeled because the starfish sperm chromatin in figure 1 is 30 - 40nm, in agreement with Woodcock's (1994) measurements of 33 - 36 nm. The scale bar in figure 4 also appears correct because the cytoplasmic particles, which I believe are ribosomes, are 20 - 25nm wide. I suggest that the authors provide a detailed figure that shows how they measure chromatin width, including controls for structures of known dimensions, like the ribosomes.
My second concern is that the cellular structure may have been grossly disrupted by the sample preparation procedure. The nuclear envelope and the E.R. membranes are absent in all the figures that show the nuclear periphery, suggesting to me that the cells have suffered extraction artifacts. Since ESI enhances phosphorus signals compared to other imaging modalities, intact membranes should be clearly seen. The absence of membrane densities diminishes my confidence in the claim that linker DNA was visualized. The authors should address why membranes were not seen and how it could impact their measurements of chromatin densities.

Other comments:
1. The thickness of the tomographic slices should be stated. Otherwise, it is difficult to measure the width of chromatin structures. For example, a 2-nm-thick slice could include "top" and "bottom" portions of 30-nm thick fibers, making them appear thinner on average.
2. In the introduction, the strengths and weaknesses of cryo-EM were not discussed at all even though key papers that employed this technique were individually cited (Woodcock, 1994; Eltsov, 2008; Nishino, 2012).
3. On page 4, the authors claim to have observed 10-nm fibers in somatic cells surrounding the starfish testis. Please provide an example figure.
4. On page 4, the term "canonical 10 nm nucleosome fiber" is used. The word "canonical" implies that the community has more or less agreed to a single model. The chromatin community hasn't even agreed on what a canonical 30-nm fiber is! I recommend deleting "canonical".

5. On page 5, the figure callout "pronounced in the MEF cells (Figure 1C)" should probably be "Figure 1B".
6. In the M&M on page 8, Naughton, 2010 is incorrectly cited. I believe the authors meant Aronova, 2007.
7. In the M&M, the Chimera software should be cited.
8. In the M&M, the specimen-level pixel size was not stated.
9. In the Figure 3B-C legend, the order of the panels is backwards.
10. In Figure 3B-C, what are the striations that run horizontally?

Referee #4:

This manuscript challenges the notion of the 30 nm fiber as an organizing principle in eukaryotic cells, and in fact posit that chromatin in many cell types consists of 10 nm fibers. There is increasing evidence for this from other labs as well using complementary approaches, and thus the study is very timely and potentially 'paradigm shifting'. I believe this should be published, with the corrections listed below. The study combines ESI with electron tomography to even allow resolution of overlapping fibers in compact chromatin domains.
The authors study chromatin organization in chromocenters (hetero chromatic) as well as in open domains, and unexpectedly find only 10 nm fibers. Under the same conditions, 30 nm fibers are observed in star fish sperm nuclei (a cell type that has been known to have higher chromatin order structures), and this is taken as a confirmation that the 30 nm fibers in MEF cells didn't get disrupted upon preparation. Two caveats here. First, given the limitations of microscopy, what is the proof that these are really 30 nm fibers? Isn't this kind of a circular argument? second, there are likely additional proteins / small molecules that promote formation of these specialized structures, and thus these could be more protected than 'normal' chromatin against preparation artifacts. Having said that, it's probably as good a control as it gets. The authors argue that individual nucleosomes can be observed by their technique; in absence of a scale bar this is impossible to verify - if the structures in fig. 3 and in the two movies indeed are nucleosomes, they are remarkably fuzzed out. This may be the case, but it would be good to visualize a defined array under these conditions so that we know how these would look like with this particular technique - can this be done, by putting a defined array into a 'cell-like' milieu?
The study goes on to analyze different cell types and show that their chromatin as well consists of 10 nm fiber and reiterate their previous notion that nucleosomes serve primarily to help the DNA fold upon itself.
Minor details
• I cannot see a scale bar in any of the pictures.
• Ditto movies - we need to have an idea of the size. It is nowhere stated how large the single nucleosomes appear.
• Figure legend 1 could be more extensive. The singular of nuclei is nucleus.

1st Revision - authors' response 30 July 2012

Referee #2:

In the manuscript entitled "Open and closed domains in the mouse genome are configured as 10 nm chromatin fibres", the authors the ongoing controversy concerning the existence of the 30 nm chromatin fiber in mouse cells. Upon careful examination of both cultured mouse embryonic fibroblasts and cells extracted from murine tissues using ESI-EM pioneered by the senior author, the authors report that they exclusively observe 10 nm fibers, even heterochromatic domains such as chromocenters. This holds true whether they examined chromatin within murine cells in culture, or within issues extracted from various organs in the mouse. Furthermore, the authors show that they can observe 30 nm fibers in starfish sperm nuclei, providing evidence that their method analysis does not disrupt putative higher order chromatin compaction. This concise report offers a valuable

insight into the chromatin conformation inside living tissues, and provides further argument to the ongoing debate of whether the 30 nm higher order chromatin fiber actually exists in mammalian cells.

However, there are 3 major concerns that should be addressed to bolster the case presented by the authors:

1) The main focus of the article is the presence or absence of a chromatin fiber of certain dimensions, but no quantitative data is provided in the manuscript. Given the excellent quality of ESI-electron tomography figures presented by the authors, and the availability of 30 nm fiber control (starfish sperm), a sample image with actual measurements should be included, alongside graphs or tables outlining the actual numbers and statistical analysis of a significant number of measurements. This would allow readers to gain a fuller appreciation of the exclusive presence of 10 nm fibers in analyzed samples.

Response: Thank you for your helpful comments. We have now included a new figure (Supplemental Figure 3) to address this concern. We have utilized a Fourier transform analysis enabling us to sample hundreds of chromatin fibres (and intervening spaces). A minimum of 70 regions were analyzed from each cell type.

2) Similarly, due to the quantitative nature of the claim, the readers must be allowed to critically evaluate each image presented in the manuscript in context of the size of fibers. This can be achieved by consistent use of clearly labeled scale bars. However, figures 1 and 4 only provide scale bar in single panels, while figures 2 and 3 omit the scale bar entirely. The authors should include clearly labeled scale bars of relevant sizes (ex. 10nm or 30nm) in each panel to let the readers easily identify the dimensions of structures presented in the figures for themselves.

Response: We have now added a 10 nm or 30 nm scale bar to the figures. We thank the reviewer for this helpful suggestion as we hope it greatly aids the reader in assigning dimensions to individual chromatin fibres.

3) A significant technical concern is that although the starfish retains its 30nm chromatin fiber under these sample preparation conditions: after all, this organism lives in harsher oceanic environmental conditions than the mouse. Mammalian nuclear membranes might be more sensitive to manipulation such as freeze/drying, so that the 30nm chromatin fiber collapses globally in mouse nuclei. The authors should provide a strong clear argument for why they don't think this is an explanation for their results. Alternatively, they should present additional data from other species (say plants, or yeast or worm etc), which also don't (or do) demonstrate 30nm fibers. This will robustly and unequivocally make the point that 30nm fibers are indeed not a dominant organizing principle in the mouse nucleus.

Response: This is indeed a very crucial point and one that we have attempted to address in the new Supplement figure (S3). Buffer conditions have a profound effect on chromatin structure and assembly *in vitro*. Although we would hesitate to rule out the possibility that variations in the nuclear salt concentration are not responsible for the unique 30 nm chromatin fibre structures in these cells, we offer that a more likely explanation would involve the unique biochemistry of the chromatin in these cells as they contain ~5% protamine. We have now highlighted a field from the mouse lymphocyte were several ribosomes are well represented in the image. These ribonuclear protein complexes have well characterized dimensions. We found that our preparation conditions had no significant effect on the measured dimensions of the ribosomes. We would argue that this provides an ideal internal standard within each sample and will alleviate this concern. Moreover, mouse cells prepared in different ways, cryo- and aldehyde fixation, exhibit analogous chromatin structures and organizations.

Minor concerns:

- *It should be clarified in the abstract that the findings of the paper show exclusive*

presence of 10nm fibers "in mouse", as that was the subject and title of the study.

Response: We have now included “in mouse” in our abstract

- *In the methods section the authors describe immunofluorescent staining using H3K9me3-specific primary antibody and Cy3-labeled secondary antibody to identify heterochromatin rich regions. The reviewer would like to see an image of such staining correlated with ESI image, showing that the region analyzed is indeed enriched in this mark, as claimed in the manuscript (Paragraph 2 of Results and Discussion).*
- *Also in paragraph 2, the authors mention the exclusive presence of 10 nm fibers in open and closed domains, and reference Figure 1. To that end, a magnification of the open chromatin domain, similar to the one provided for closed domain, would be helpful.*

Response: We have now included as a part of Supplemental Figure 1 a series of panels that we hope will satisfy these two concerns. We have shown an example of a physical section labeled on the section with an H3K9me3 antibody. We then collected and processed tomograms from a region that was enriched and a region depleted of the heterochromatin histone modification from this same physical section.

- *For clarity, supplemental figures should be referenced in the manuscript in order, i.e. Supplemental Figure 1 before Supplemental Figure 2.*

Response: Thank you. We have corrected this oversight and Supplemental Figure 1 is now referred to before 2.

- *On page 4, the authors mention 30 nm chromatin fiber in starfish sperm nuclei, and reference Supplemental Figure 2A-C. However, these figures are of mouse cells, namely mouse embryonic fibroblasts, lymphocytes, and liver cells, according to the figure legend. Did the authors mean Figure 1C of the manuscript instead?*

Response: We did indeed. Thank you and this has now been corrected

- *Figure 3 legend is misleading. Digitally zoomed images are on the right (not left), and side views are on the left (not right).*

Response: We have now corrected this confusing right-left mix up in the figure legend.

- *On page 5 the authors mention that lymphocytes have high representation of compact chromatin domains throughout the nucleus. This should be supported with an appropriate reference. Likewise, the mention of equal representation of dispersed and compact chromatin domains in liver cells should also be accompanied by a reference.*

Response: We have now added a reference (Rapkin et al 2012) to support these claims. Although, the authors would like to disclose, that these supporting data are derived from data generated in the Bazett-Jones lab.

- *On page 5 the authors discuss open and compact chromatin domains in MEF cells, but reference Figure 1C, which shows starfish sperm nucleus.*

Response: We have corrected the incorrectly cited panel in the text and it now reads (1B).

- *The claim in the last paragraph that "interconversion of 10 nm to 30 nm chromatin fibres does not represent a major gene regulatory mechanism, and is not required for regulation" should be supported by an appropriate reference.*

Response: This claim is one that is generated by our interpretation of these data presented in this paper. A prevailing model is that 10-30 nm transitions represents a major regulatory step. Since we were unable to detect a 30 nm fibre we argue that this

conversion is unnecessary for genome regulation.

- *(Optional) The authors mention they observed 10nm fibers in the somatic cell nuclei of starfish testis. Can the authors show an image of that?*

Response: Unfortunately we were unable to obtain examples of non-germline cells (somatic) and mature sperm in the same sections. Given the time constraints of the revision process we are unable to provide this result.

Referee #3:

Chromatin structure is important to many biological problems and is of interest to a broad audience. The 30-nm fiber models of chromatin folding have been accepted by most cell biologists. In the past few years, however, the generality of the 30-nm-fiber has been challenged by studies of chromatin structure in situ. In this paper, the authors used electron spectroscopic imaging in combination with tomography to study the 3-D organization of chromatin in mammalian cells. ESI tomography is a novel and powerful technique because it provides 3-D chemical information, namely the distribution of the cell's phosphorus-rich components. The authors conclude from their image analysis that the organization of both "open" and "closed" chromatin is the "beads-on-a-string" 10-nm fiber - not the 30-nm fiber. From an electron microscopy point of view, the most significant claim is that the path of linker DNA could be determined. Therefore, the authors' present work could be a rich platform for future studies on chromatin folding and transcriptional regulation in situ.

My initial enthusiasm was curbed, however, by two major concerns. First, how was the chromatin width measured? The nucleosome is ~10 nm in diameter, so I would expect the ESI densities to be 8 - 12 nm wide at maximum, taking into account inaccuracies in magnification calibration. To my surprise, I consistently measured the chromatin dimensions to be 15 - 25nm using the authors' scale bars in figures 1 and 4. The scale bar in figure 1 appears to be correctly labeled because the starfish sperm chromatin in figure 1 is 30 - 40nm, in agreement with Woodcock's (1994) measurements of 33 - 36 nm. The scale bar in figure 4 also appears correct because the cytoplasmic particles, which I believe are ribosomes, are 20 - 25nm wide. I suggest that the authors provide a detailed figure that shows how they measure chromatin width, including controls for structures of known dimensions, like the ribosomes.

Response: We have now added 10 or 30 nm scale bars to figures where appropriate and hope this will aid in the reviewers' (and general readers') ease in making direct measurements on chromatin fibres of interest. There are indeed some chromatin fibres, whereby overlapping in the z-dimension, appear much thicker than the average fibre width (9-12 nm). We have now provided a high resolution and digitally enhanced image of ribosomes after sample preparation and tomographic reconstruction and find their dimensions to be very consistent with those derived from alternative biophysical methodologies (21-23 nm). We have illustrated an example of this in the new figure (Supplemental Figure 3). In addition to the manual measurements made in our first submission, we have now included a Fourier transform analysis, which enabled the measurement of hundreds of chromatin fibres (and inter-fibre spaces). We hope this extensive analysis and new figure will satisfy this concern.

My second concern is that the cellular structure may have been grossly disrupted by the sample preparation procedure. The nuclear envelope and the E.R. membranes are absent in all the figures that show the nuclear periphery, suggesting to me that the cells have suffered extraction artifacts. Since ESI enhances phosphorus signals compared to other imaging modalities, intact membranes should be clearly seen. The absence of membrane densities diminishes my confidence in the claim that linker DNA was visualized. The authors should address why membranes were not seen and how it could impact their

measurements of chromatin densities.

Response: Lipid membranes can only be visualized by conventional transmission electron microscopy by post-fixation with Osmium tetroxide and uranyl or lead heavy atom stains. We do not use these in ESI since they are not required to generate contrast and can interfere with quantitative elemental maps.

Other comments:
1. The thickness of the tomographic slices should be stated. Otherwise, it is difficult to measure the width of chromatin structures. For example, a 2-nm-thick slice could include "top" and "bottom" portions of 30-nm thick fibers, making them appear thinner on average.

Response: We have now added the slice dimensions to the Materials and Methods section. Slices were averaged from 5 slices and the total volume measured was approximately 13 nm. These slices were used to generate the images shown and to make accurate three-dimensional measurements of chromatin fibres.

2. In the introduction, the strengths and weaknesses of cryo-EM were not discussed at all even though key papers that employed this technique were individually cited (Woodcock, 1994; Eltsov, 2008; Nishino, 2012).

Response: Cryo-EM is outstanding for imaging isolated chromatin structures. However, *in situ* chromatin measurements have some serious limitations. We have added the following to the introduction of the manuscript at the beginning of page 3 "Cryo electron microscopy (cryoEM) overcomes some limitations of conventional TEM, retaining chromatin in a hydrated environment. Recent studies by cryoEM methods have been used to address the chromatin fibre configuration in metaphase chromosomes, and have provided high-resolution images of extracted chromatin. The cryoEM approach has not yet addressed the question of the chromatin fibre configuration in intact interphase cells primarily due to relatively low contrast of chromatin *in situ* in interphase nuclei."

3. On page 4, the authors claim to have observed 10-nm fibers in somatic cells surrounding the starfish testis. Please provide an example figure.

Response: Unfortunately these cells in this sample are extremely rare and given the time constraints of the revision process we were unable to do so.*Please also see response to referee 2.

4. On page 4, the term "canonical 10 nm nucleosome fiber" is used. The word "canonical" implies that the community has more or less agreed to a single model. The chromatin community hasn't even agreed on what a canonical 30-nm fiber is! I recommend deleting "canonical".

Response: We agree with the reviewer on this point and have removed the term "canonical".

5. On page 5, the figure callout "pronounced in the MEF cells (Figure 1C)" should probably be "Figure 1B".

Response: Thank you. We have corrected this mistake in the text on page 5.

6. In the M&M on page 8, Naughton, 2010 is incorrectly cited. I believe the authors meant Aronova, 2007.

Response: We did indeed. Thank you for noting this oversight and this reference has been corrected.

7. In the M&M, the Chimera software should be cited.

Response: Thank you. We have now included the Pettersen et al 2004 reference

8. In the M&M, the specimen-level pixel size was not stated.

Response: The pixel size is 2.56 nm in the images in Figures 1 and 4 and 1.3 nm in figures 2-3. We have now added this detail to the Materials and Methods section.

9. In the Figure 3B-C legend, the order of the panels is backwards.

Response: Thank you. We have corrected this figure legend.

10. In Figure 3B-C, what are the striations that run horizontally?

Response: The striations are the result of viewing cubic pixels on an angle. Visualization programs sometimes have problems displaying density smoothly and rendering without these artifacts.

Referee #4:

This manuscript challenges the notion of the 30 nm fiber as an organizing principle in eukaryotic cells, and in fact posit that chromatin in many cell types consists of 10 nm fibers. There is increasing evidence for this from other labs as well using complementary approaches, and thus the study is very timely and potentially 'paradigm shifting'. I believe this should be published, with the corrections listed below. The study combines ESI with electron tomography to even allow resolution of overlapping fibers in compact chromatin domains.
The authors study chromatin organization in chromocenters (hetero chromatic) as well as in open domains, and unexpectedly find only 10 nm fibers. Under the same conditions, 30 nm fibers are observed in star fish sperm nuclei (a cell type that has been known to have higher chromatin order structures), and this is taken as a confirmation that the 30 nm fibers in MEF cells didn't get disrupted upon preparation. Two caveats here. First, given the limitations of microscopy, what is the proof that these are really 30 nm fibers? Isn't this kind of a circular argument? second, there are likely additional proteins / small molecules that promote formation of these specialized structures, and thus these could be more protected than 'normal' chromatin against preparation artifacts. Having said that, it's probably as good a control as it gets. The authors argue that individual nucleosomes can be observed by their technique; in absence of a scale bar this is impossible to verify - if the structures in fig. 3 and in the two movies indeed are nucleosomes, they are remarkably fuzzed out. This may be the case, but it would be good to visualize a defined array under these conditions so that we know how these would look like with this particular technique - can this be done, by putting a defined array into a 'cell-like' milieu?

Response: Thank you for your helpful comments. We agree with you that the issue of an appropriate control for these experiments was not a simple issue. We too think that the starfish sperm chromatin is as good as it can get. As for mimicking the cell milieu in a reconstituted system, many studies have attempted to do so. Modest variations in salt concentrations affect chromatin structures under these conditions and are largely responsible for the ambiguity surrounding the question of chromatin structure and assembly. We have added additional scale bars to figures where appropriate and hope that this will provide an easy metric with which readers may measure fibres of particular interest. The nucleosomes highlighted in Figure 3 are indeed “fuzzy”. We point out that they were imaged in the context of a complex concentrated environment of the cell, and many processes *in vivo* are likely responsible for perturbing the compact structures of the nucleosome observed by other biophysical methods, including ESI of purified nucleosomes. We think that being able to visualize individual nucleosomes at all in three dimensions *in situ* is a major accomplishment. Also, crystal structures of nucleosomes

represent averages of thousands of individual molecules, which eliminate the variations observed in these molecular complexes.

The study goes on to analyze different cell types and show that their chromatin as well consists of 10 nm fiber and reiterate their previous notion that nucleosomes serve primarily to help the DNA fold upon itself.

Minor details

• *I cannot see a scale bar in any of the pictures.*

Response: We have added additional scale bars to our figures.

• *Ditto movies - we need to have an idea of the size. It is nowhere stated how large the single nucleosomes appear.*

Response: We have now added a figure bar to our movies. The bar changes in length throughout the movie as the zoom on the feature of interest changes and as the chromatin is tilted.

• *Figure legend 1 could be more extensive. The singular of nuclei is nucleus.*

Response: Thank you. We have corrected and extended the figure legend.

3rd Editorial Decision 16 August 2012

Thank you for the submission of your revised manuscript. As you will see, while all referees support publication of the study in our journal now, both referees 2 and 3 still have minor concerns that need to be addressed in the manuscript text before we can proceed with the official acceptance.

I also would like to suggest slight modifications to the abstract as follows:

The mammalian genome is compacted to fit within the confines of the cell nucleus. DNA is wrapped around nucleosomes forming the classic "beads-on-a-string" 10 nm chromatin fibre. 10 nm chromatin fibres are thought to condense into 30 nm fibres. This structural reorganization is widely assumed to correspond to transitions between active and repressed chromatin, thereby representing a major regulatory event. Here, by combining electron spectroscopic imaging with tomography, three-dimensional images are generated, revealing that both open and closed chromatin domains in mouse somatic cells are comprised of 10 nm fibres. These findings indicate that the 30 nm chromatin model does not reflect the true regulatory structure in vivo.

Please let me know if you disagree with these changes.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #2:

In this revised version of the article by Bazett Jones and colleagues, I appreciate the additional work the authors have put into improving the manuscript and addressing all previous concerns. The clarity of the figures has been greatly enhanced by the addition of properly sized scale bars. Also the logic and workflow of numerical analysis of the data, as clearly laid out in Supplemental Figure 3, provides a very convincing argument for the exclusive presence of 10 nm chromatin fibers in mouse cells and tissues. As stated previously, this report is a valuable addition to the ongoing debate about the existence of 30 nm fibers in situ in mammalian cells, and as such would be of great interest to the chromosome biology field.
However, as much as the Fourier analysis underlines the findings in the mouse cells, it raises concerns when applied to starfish sperm nuclei. It seems to me that the FT shows 20 nm chromatin fibers in those cells, with highly regular 43 nm periodicity, rather than 30nm fibers. This is in agreement with the data presented in Figure 1D, but questions the reliability of the method of analysis, since starfish sperm nuclei cells were specifically used as 30 nm fiber control. Since "known" 30 nm fibers measure as 20 nm following the ESI tomography and Fourier analysis (unless these control 30nm fibers are truly not 30nm at all), what does that say about the chromatin fibers found in mouse cells, which measure 10 nm? Wouldn't that indicate their "actual" dimensions are closer to ~15 nm? Furthermore, the authors do not explain this result (i.e. starfish), nor do they refer to it anywhere in the text of the manuscript. The reviewer strongly believes that the results presented in the Supplemental Figure 3E, especially those concerning 30 nm fibers in starfish sperm nuclei, should be clearly summarized and MUST be explained in the body of the manuscript before it is accepted for publication. I strongly feel this is necessary so that lay readers are not confused (and subsequently skeptical) as to why 30 nm fibers appear as 20 nm structures in this manuscript.

Minor comment:
The reviewer appreciates the inclusion of H3K9me3 staining in Supplemental Figure 1A. If magnifications of the "open" (Sup. 1B) and "closed" (Sup. 1C) chromatin domains were taken from the panel Sup. 1A, it would be of benefit to the reader to indicate which areas of the image where magnified. It would show how the enhanced areas are "rich" or "poor" in H3K9me3 staining.

Referee #3:

The authors have addressed most of my concerns, and are commended for their new figure showing how chromatin spacings were measured. Regarding the visualization of lipid membranes, I believe the authors misunderstood my question and should be given a chance to address this point, which will be of interest to many readers.

Response: Lipid membranes can only be visualized . . .

In the present study, shouldn't the ESI tomography enable the visualization of the phosphate head groups in the unstained lipid bilayers that constitute the nuclear envelope and endoplasmic reticulum? I would expect, perhaps naively, that the membranes might appear as two thin lines corresponding to the densely packed head groups of opposite leaflets. Instead, the tomograms do not have any structures that resemble membranes. Resolution and contrast should not be limiting factors because the phosphate signal from both the gyres of nucleosome DNA and the linker DNA could also be visualized. Since the authors claim that cellular structure has not been grossly perturbed, can they offer an explanation for why the contrast mechanism of ESI tomography can reveal unstained DNA but not unstained membranes? Or is it too early to say given the novelty of ESI?

Referee #4:

good to go

2nd Revision - authors' response 21 August 2012

Thank you for the submission of your revised manuscript. As you will see, while all referees support publication of the study in our journal now, both referees 2 and 3 still have minor concerns that need to be addressed in the manuscript text before we can proceed with the official acceptance.

I also would like to suggest slight modifications to the abstract as follows:

The mammalian genome is compacted to fit within the confines of the cell nucleus. DNA is wrapped around nucleosomes forming the classic "beads-on-a-string" 10 nm chromatin fibre. 10 nm chromatin fibres are thought to condense into 30 nm fibres. This structural reorganization is widely assumed to correspond to transitions between active and repressed chromatin, thereby representing a major regulatory event. Here, by combining electron spectroscopic imaging with tomography, three-dimensional images are generated, revealing that both open and closed chromatin domains in mouse somatic cells are comprised of 10 nm fibres. These findings indicate that the 30 nm chromatin model does not reflect the true regulatory structure in vivo.

Please let me know if you disagree with these changes.

Response: We think these revisions improve the abstract.

Referee #2:

In this revised version of the article by Bazett-Jones and colleagues, I appreciate the additional work the authors have put into improving the manuscript and addressing all previous concerns. The clarity of the figures has been greatly enhanced by the addition of properly sized scale bars. Also the logic and workflow of numerical analysis of the data, as clearly laid out in Supplemental Figure 3, provides a very convincing argument for the exclusive presence of 10 nm chromatin fibers in mouse cells and tissues. As stated previously, this report is a valuable addition to the ongoing debate about the existence of 30 nm fibers in situ in mammalian cells, and as such would be of great interest to the chromosome biology field.

However, as much as the Fourier analysis underlines the findings in the mouse cells, it raises concerns when applied to starfish sperm nuclei. It seems to me that the FT shows 20 nm chromatin fibers in those cells, with highly regular 43 nm periodicity, rather than 30nm fibers. This is in agreement with the data presented in Figure 1D, but questions the reliability of the method of analysis, since starfish sperm nuclei cells were specifically used as 30 nm fiber control. Since "known" 30 nm fibers measure as 20 nm following the ESI tomography and Fourier analysis (unless these control 30nm fibers are truly not 30nm at all), what does that say about the chromatin fibers found in mouse cells, which measure 10 nm? Wouldn't that indicate their "actual" dimensions are closer to ~15 nm? Furthermore, the authors do not explain this result (i.e. starfish), nor do they refer to it anywhere in the text of the manuscript. The reviewer strongly believes that the results presented in the Supplemental Figure 3E, especially those concerning 30 nm fibers in starfish sperm nuclei, should be clearly summarized and MUST be explained in the body of the manuscript before it is accepted for publication. I strongly feel this is necessary so that lay readers are not confused (and subsequently skeptical) as to why 30 nm fibers appear as 20 nm structures in this manuscript.

Response: Thank you for these helpful comments that we think have improved the quality of the manuscript and should aid in the interpretation of our data for the lay reader. We were also very intrigued by the fibre diameter results of the starfish chromatin. Because of the 5% protamine content, these so-called 30 nm fibres may differ from the canonical 30 nm fibres studied in cell-free or partially extracted chromatin conditions. Nevertheless, in the literature we have noted that the so-called 30 nm fibre ranges in size between 20-30 nm. In Fourier space we measure these fibres at 24 nm, consistent with the measurements of made from the tetranucleosome crystal structure of the “30 nm fibre”. We have now added a more in-depth discussion of this issue to the Results and Discussion section of the manuscript. On page 4: “In real space, measurements of fibre diameter ranged from 20-30 nm, consistent with both x-ray scattering measurements (Langmore & Paulson, 1983) and conventional TEM (Horowitz et al, 1994).” And at the bottom of page 7: “We also observe a 20 nm Fourier transform peak and a very strong 43 nm peak. Comparing the Fourier

transform with real space measurements (described earlier), we conclude that in the starfish sperm the fibre-fibre spacing is approximately 20 nm."

Minor comment:
The reviewer appreciates the inclusion of H3K9me3 staining in Supplemental Figure 1A. If magnifications of the "open" (Sup. 1B) and "closed" (Sup. 1C) chromatin domains were taken from the panel Sup. 1A, it would be of benefit to the reader to indicate which areas of the image where magnified. It would show how the enhanced areas are "rich" or "poor" in H3K9me3 staining.

Response: We have now included boxes around the regions in the low magnification image marked with the H3K9me3-enriched regions to indicate the regions from which the tomograms were generated.

Referee #3:

The authors have addressed most of my concerns, and are commended for their new figure showing how chromatin spacings were measured. Regarding the visualization of lipid membranes, I believe the authors misunderstood my question and should be given a chance to address this point, which will be of interest to many readers.

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Response: We have now added a section to the Supplemental Materials and Methods section addressing the issue of phospholipid visualization by ESI. We hope this will clarify this concern.

"ESI is capable of detecting the phosphorus signal contributed by the phospholipid bilayer *in situ* only when an osmium post-fixation step is used, where osmium tetroxide crosslinks the bilayer's protein and lipid components. Because this fixation step was not required in our study, the phospholipids were extracted during dehydration, and therefore were not visualized."

Referee #4:

good to go

Response: Thanks!

4th Editorial Decision 28 August 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to

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Yours sincerely,

Editor
EMBO Reports

